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## Dakin solution alters macrophage viability and function



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### ABSTRACT

**Background:** Macrophages are important in wound defense and healing. Dakin's solution (DS), buffered sodium hypochlorite, has been used since World War I as a topical antimicrobial for wound care. DS has been shown to be toxic to host cells, but effects on immune cells are not well documented.

**Materials and methods:** DS at 0.5%, 0.125%, and ten fold serial dilutions from 0.25% 0.00025% were evaluated for cellular toxicity on murine macrophages (J774A.1). The effect of DS on macrophage adhesion, phagocytosis, and generation of reactive oxygen species was examined. Macrophage polarization following DS exposure was determined by gene expression using quantitative real time polymerase chain reaction.

**Results:** Concentrations of DS >0.0025% reduced macrophage viability to <5% in exposure times as short as 30 s. Similarly, phagocytosis of *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Aspergillus flavus* were significantly reduced at all tested concentrations by macrophages pretreated with DS. H<sub>2</sub>O<sub>2</sub> production was reduced by 8% 38% following treatment with 0.00025% 0.125% DS. Macrophage adherence was significantly increased with >0.0025% DS after 15 min of exposure compared with controls. Quantitative real time polymerase chain reaction demonstrated that DS exposure resulted in classical macrophage activation, with increased expression of inducible nitric oxide synthase 2, interferon  $\gamma$ , and interleukin (IL) 1 $\beta$ .

**Conclusions:** DS at clinically used concentrations (0.025% 0.25%) was detrimental to macrophage survival and function. For optimal clinical use, understanding the impact of DS on macrophages is important as depletion may result in impaired pathogen clearance and delayed healing. These findings indicate that 0.00025% DS is a safe starting dose; however, optimal use of DS requires further validation with *in vivo* models.

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## 1. Introduction

Sodium hypochlorite has broad antimicrobial activity against bacteria, fungi, viruses, and parasites [1]. Dakin's solution (DS) is buffered sodium hypochlorite, which has a long history of use as a topical antiseptic in war wounds. Henry Dakin first advocated the use of sodium hypochlorite as a topical anti-septic during World War I [2]. Dakin worked closely with Alexis Carrel, a Nobel prize winning surgeon, who popularized the use of DS in his work published in 1917, titled "The Treatment of Infected Wounds" [3,4]. During World War II, Bunyan began using sodium hypochlorite in the management of burns [5].

Interest in the use of DS has been renewed in the United States military given the recognition of and difficulty in treating invasive fungal infections in soldiers who sustained blast trauma with lower extremity amputations in Operation Enduring Freedom [6]. Invasive fungal infections have also been seen in trauma associated with agricultural, motor vehicle, and blunt crush injuries, and during natural disasters as recently reported among tornado victims [7–9]. Fungal infections in burn injury are also important as the incidence of fungal infections documented in the literature ranges from 6.3%–44% [10].

Currently, DS is available commercially at full strength (0.5%), half strength (0.25%), quarter strength (0.125%), and 1/40 strength (0.0125%). Concerns have been raised about the modern use of DS as the product information for all four dilutions are similar: once daily for lightly to moderately exudative wounds, and twice daily for heavily exudative wounds or highly contaminated wounds [3]. In addition, the efficacy and safety of DS has been debated since Alexander Fleming first raised concerns in 1919 [11]. Since that time, there have been numerous studies on DS and other chlorite containing antiseptics. *In vitro* studies have revealed diminished fibroblast viability, decreased fibroblast migration, decreased keratinocyte viability, decreased keratinocyte growth rates, and decreased endothelial cell viability [12–19]. In addition, a negative impact on neutrophil viability and neutrophil migration have been described [16,20].

Recently, our research group demonstrated that DS exhibited dose dependent toxicity and efficacy (against four clinical mold isolates including *Aspergillus* and *Mucor*) with 0.00025% appearing to optimize these parameters [21]. There is a paucity of data regarding the impact of DS on macrophage viability and function with two studies revealing decreased macrophage adherence with 5.25% DS [22,23]. Neutrophils are typically thought of as the most important leukocyte in the wound as they are the predominant cell type in the first 24 h following injury and are the primary cells responsible for bacterial clearance [24]. In comparison with neutrophils, macrophages are longer lived, produce high levels of pro inflammatory mediators that amplify immune cell recruitment and/or activation cascades, and exhibit potent phagocytic and antimicrobial effects [24]. Macrophages display two opposite phenotypes known as classically activated (M1) and alternatively activated (M2) macrophages [25]. The M1 macrophage is pro inflammatory and is often associated with tissue injury and inflammation, whereas the M2 macrophage is associated with tissue repair and fibrosis [25]. In the inflammatory phase of wound healing, invading

macrophages serve as antigen presenting cells to T cells, and kill bacteria through multiple mechanisms providing innate immunity against filamentous fungi via phagocytosis of conidia [24,26–28]. Classic wound models view wound healing as being primarily directed by cytokines and growth factors, but it is becoming apparent that macrophages are key orchestrators of the wound healing process [26].

In summary, macrophages are dynamic cells that are important in the wound microenvironment as they mediate pathogen defense and the healing cascade [25–28]. Thus, in this study we sought to characterize the impact of DS on macrophage viability and function *in vitro*.

## 2. Materials and methods

### 2.1. Cell lines and reagents

Murine macrophages, (J77A.1; ATCC TIB 67), isolated from adult females and purchased from ATCC were used in this study (Manassas, VA) were grown in Dulbecco modified eagle medium (DMEM) supplemented with 10% fetal bovine serum, 10 U/mL penicillin, 10 µg/mL streptomycin, and maintained at 37°C in 5% CO<sub>2</sub>. DS (buffered sodium hypochlorite) was purchased from Century Pharmaceuticals Inc (Indianapolis, IN) at full strength (0.5%), half strength (0.25%), quarter strength (0.125%), and 1/40 strength (0.0125%).

### 2.2. Clinical strains and growth conditions

Bacterial strains used included *Staphylococcus aureus* strain UAMS 1 (ATCC strain 49,230), a well characterized methicillin susceptible osteomyelitis isolate, and *Pseudomonas aeruginosa* strain PAO1 [29,30]. *Aspergillus flavus*, strain SAMMC 1, is a clinical isolate selected from a strain collection from the San Antonio Military Medical Center, Ft. Sam Houston, TX. This isolate was collected as a part of patient care and not directly related to clinical research. *S. aureus* and *P. aeruginosa* were grown in tryptic soy broth or Luria–Bertani broth, respectively, overnight at 37°C with agitation. A *flavus* was subcultured on potato flake agar slants at 35°C. Conidia were harvested by washing 7 d old slant culture with phosphate buffered saline (PBS) containing 0.1% Tween20, filtered through a 40 µm pore size cell strainer to separate conidia from mycelium, and stored in DMEM at 4°C.

### 2.3. Cellular viability assays

Effect of DS on cellular viability was performed as previously described [21]. Briefly, macrophages were seeded into 96 well black plates with clear bottoms (Fisher, Pittsburgh, PA), grown to confluence, and exposed to 0.00025, 0.0025, 0.025, 0.125, 0.25, and 0.5% DS diluted in saline (0.9% NaCl, pH 7.4) for 0.5, 1, 5, 10, and 15 min at the designated concentrations. Time points were selected based on previous studies demonstrating significant cellular toxicity on various cell lines after exposure to DS for <30 min. After treatment cells were washed, resuspended (PBS; pH 7.4), and cell viability was measured using the CellTiter Fluor assay (Promega, Madison, WI). Cell viability

was reported as a percentage to untreated controls. For analysis of macrophage function following treatment and to accurately control exposure times of DS, cells were exposed to an equal volume of 0.2 M sodium thiosulfate (Sigma-Aldrich, St. Louis, MO), a neutralizing agent, immediately following treatment.

#### 2.4. Phagocytosis assays

Cells ( $5 \times 10^5$  cells) were seeded into 24 well tissue culture plates. For the bacterial phagocytosis assays, cells were infected with 10 multiplicity of infection (MOI) of *S aureus* UAMS 1 or with 50 MOI of *P aeruginosa* PAO1 in 500  $\mu\text{L}$  DMEM per well for 30 min. After exposure, cells were washed with PBS, incubated for 2 h in medium containing 100  $\mu\text{g}/\text{mL}$  gentamicin, to kill extracellular bacteria, washed, and then lysed with 0.1% Triton X 100. The number of internalized bacteria was determined by plating serial dilutions of lysates onto blood agar plates. The bacterial phagocytosis index was calculated by using the following formula: (bacteria recovered [colony forming units/mL]/bacteria inoculated [colony forming units/mL]). [31]. The fungal phagocytic index was determined as previously described [32]. Before use within the phagocytosis assays, fungal conidia were labeled with FUN 1 cell stain (10  $\mu\text{M}$ ; Invitrogen, Grand Island, NY) as recommended by the manufacturer. Macrophages were infected as mentioned previously with 2 MOI of *A. flavus* in 500  $\mu\text{L}$  DMEM per well for 2 h. After exposure, cells were fixed with 4% paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA) and counter stained with calcofluor white (Invitrogen). External and internalized conidia were distinguished by fluorescence microscopy by distinguishing between calcofluor white, which binds to fungal cell wall components but does not penetrate macrophage cell membrane and FUN 1 stain. Phagocytosed conidia appear green (FUN 1 $^+$ ), whereas conidia external to macrophages (i.e., not phagocytosed) appear blue under fluorescence microscopy. Two hundred cells per slide were counted by fluorescence microscopy. Phagocytic index is the average number of conidia phagocytosed by macrophages that were observed by fluorescence microscopy to contain at least one internalized conidium.

#### 2.5. Measurement of reactive oxygen species (ROS) release

Release of ROS was evaluated by measuring the extracellular production of  $\text{H}_2\text{O}_2$  following lipopolysaccharide (LPS) stimulation in macrophages using the Amplex Red fluorometric assay (Molecular Probes, Carlsbad, CA) as recommended by the manufacturer. Briefly, cells were exposed to DS as mentioned previously, followed by neutralization in sodium thiosulfate, resuspended in PBS, and plated into black clear bottom 96 well plate at a density of  $10^5$  cells per well. Treated cells were then stimulated with 100 ng/mL of LPS for 1 h, followed by the addition of Amplex Red (20  $\mu\text{M}$ ) and horse radish peroxidase (0.1 U/mL), and incubated for 37°C for 30 min. After incubation, supernatants were transferred to a 96 well plate and the fluorescence was measured (Ex 540 nm, Em 590 nm). Concentrations of  $\text{H}_2\text{O}_2$  in supernatants were calculated using a standard curve generated with  $\text{H}_2\text{O}_2$ . Cell

cultures were lysed in 0.1% Triton X 100 and protein concentrations were determined using a bicinchoninic acid assay. Total  $\text{H}_2\text{O}_2$  concentrations were normalized to cellular protein concentrations. For controls, cells in DMEM were washed with PBS and exposed to a neutralizing agent as described previously.

#### 2.6. Macrophage adhesion assay

Macrophage adhesion assays were performed as previously described with minor modifications [33]. Briefly, cell suspensions of  $2.5\text{--}5.0 \times 10^6$  cells/mL were pulsed with test concentrations of DS and neutralized with an equal volume of 0.2 M sodium thiosulfate. As a control, cells were treated with 1X PBS followed by neutralization as mentioned previously. After treatment, cells were harvested, washed with PBS, and resuspended in Roswell Park Memorial Institute medium. Viable cells after treatment were determined using a Countess Automated Cell Counter per manufacturer's instructions (Life Technologies, Grand Island, NY). Cells were then resuspended to equivalent cell densities and 200  $\mu\text{L}$  were placed into individual wells of a 48 well plate. Cells were allowed to adhere to plastic surfaces for 15 and 30 min at 37°C in 5% CO<sub>2</sub>. These timepoints were chosen as they have been cited as optimal time for adhesion [33]. After incubation, nonadherent cells were collected by aspirating media and adherent cells were collected after treatment with 0.25% Trypsin EDTA (Invitrogen, Carlsbad, CA) to calculate the total number of macrophages. Adherence index (AI) was calculated by the following equation.

$$\text{AI} = 100 \left( \frac{\text{Nonadherent macrophages/mL}}{\text{Total macrophages/mL}} \right) \times 100$$

#### 2.7. RNA extraction and quantitative real-time PCR

Cells were grown to confluence in T25 culture flasks and treated as mentioned previously at the indicated concentrations. Cells were washed, resuspended in DMEM, and incubated for 10, 30, and 60 min postexposure at 37°C in 5% CO<sub>2</sub>. RNA was extracted and purified using the RNAeasy Mini Kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. First strand synthesis was achieved with Super Script III first strand synthesis supermix with oligo dT primers (Invitrogen) for each RNA sample following recommended protocols using a PTC 100 Thermal Cycler (GMI Inc, Ramsey, MN). To examine the impact of DS on macrophage polarization, genes associated with classical macrophage activation (interleukin [IL] 1 $\beta$ , Nos2, and interferon  $\gamma$ ) and alternative macrophage activation (Arg 1, IL 10, and IL 4) were evaluated. For genes of interest, quantitative real time polymerase chain reaction was performed using a Bio Rad C1000 system and analyzed using iQ5 software (BioRad, Hercules, CA). Forward and reverse primers sets used in this study were purchased from SA Biosciences (Valencia, CA) and used according to the manufacturer recommended protocols. Amplification reactions were performed using qPCR iQSYBR Green Super Mix (BioRad) with the following conditions: 10 min at 95°C, followed by 40 cycles at 95°C for 10 s, 60°C for 30 s, and 72°C for 30 s. Three independent biological experiments with

three technical replicates were performed for each reaction. Transcript levels were normalized to the internal control, glyceraldehyde 3 phosphate dehydrogenase, and messenger RNA, and changes in relative expression were calculated using  $2^{-\Delta Ct}$  method.

## 2.8. Statistical analysis

All experiments were performed in triplicate with a minimum of three technical replicates. For multigroup comparisons, a one way analysis of variance with a Dunnett post hoc test was used to compare all treatment groups with the control group. Multiple linear regression was used to determine whether DS concentration or exposure time best predicted macrophage toxicity. Concentrations and exposure times were input into the generalized linear model with reciprocal link function using cell viability as the independent variable (JMP version 9.0.0). P values of  $<0.05$  were considered to be statistically significant.

## 3. Results

### 3.1. Effects of DS on macrophage viability

Concentrations of DS  $>0.0025\%$  exhibited significant cytotoxicity, reducing viability to below 5% in exposure times as short as 30 s (Table 1). In contrast, DS concentrations of  $\leq 0.0025\%$  exhibited minimal cellular toxicity at early time points, with toxicity increasing over time. Based on these assays, for all additional studies concentrations of  $\leq 0.125\%$  DS (representing the plateau of maximal cytotoxicity) were used to evaluate the impact on macrophage function. Multiple linear regression revealed that DS concentration ( $P < 0.0001$ ) rather than exposure time ( $P = 0.91$ ) was a significant predictor of macrophage toxicity *in vitro*.

### 3.2. Exposure of macrophages to DS reduces phagocytosis

Similar to the effects of DS on cellular viability, preexposure of macrophages to DS, at all tested concentrations, negatively impacted the ability of macrophages to phagocytize bacteria and fungal conidia (Fig. 1). For bacterial phagocytosis, preexposure of macrophages resulted in significant decreases, as indicated by the decrease in the average phagocytic index for

*S. aureus* (0.06–0.37) and *P. aeruginosa* (0.002–0.17) compared with the control group. Although preexposure to DS at all concentrations resulted in significant decreases in phagocytic activity, the largest decreases were observed after preexposure to DS  $>0.00025\%$ . As observed for bacterial phagocytosis, preexposure of macrophages to DS resulted in significant decreases in the average number of internalized fungal conidia per cell that is, decrease in the fungal phagocytic index.

### 3.3. Impaired ROS production in macrophages after exposure to DS

Treatment of macrophages with DS alone resulted in minimal production of ROS (detected as H<sub>2</sub>O<sub>2</sub>) necessitating the use of stimulation with bacterial LPS (data not shown). Compared with control groups, exposure of macrophages to DS  $\geq 0.0025\%$  significantly reduced the amount of measurable H<sub>2</sub>O<sub>2</sub> in cell supernatants following LPS stimulation (Fig. 2). The effect of DS, as for phagocytosis and viability, was observed to occur in a dose dependent manner, reducing production of H<sub>2</sub>O<sub>2</sub> by 8%, 26%, 36%, and 38% after treatment with 0.00025–0.125% of DS, respectively.

### 3.4. Effects of DS on macrophage adhesion

Exposure of macrophages to DS at all the tested concentrations did not affect macrophage adhesion to polystyrene. AI values calculated for macrophages treated with sodium thiosulfate (control) or with different DS dilutions for 15 and 30 min are shown in Table 2. The AI of macrophages treated with the higher concentrations of DS ( $\geq 0.0025\%$ ) significantly increased, reaching a maximum 66.7 and 44.4, whereas lower concentrations ( $<0.0025\%$ ) were slightly reduced (12 and 5 for 0.0025 and 0.00025% DS, respectively) compared with control (18) at 15 min. Notably, the adherence capacity of macrophages exposed to DS, with the exception of 0.025%, were significantly increased ( $P < 0.01$ ) compared with the control group.

### 3.5. Effects of DS on macrophage polarization

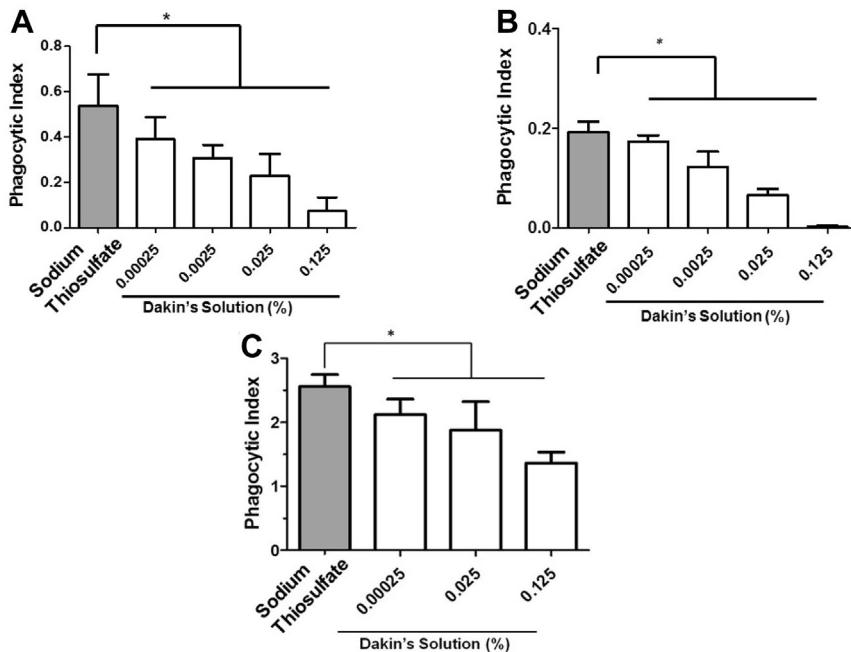
Exposure of macrophages to DS  $\leq 0.0025\%$  resulted primarily in the activation of the proinflammatory (classically activated, M1) and not the alternative (M2) macrophage phenotype (Fig. 3 and Supplemental Table 1). Gene expression analysis by

**Table 1 – Macrophage viability following treatment with the sodium thiosulfate (Na<sub>2</sub>S<sub>2</sub>O<sub>3</sub>) control or DS.**

Concentration	0.5 min <sup>*</sup>	1 min <sup>*</sup>	5 min <sup>*</sup>	10 min <sup>*</sup>	15 min <sup>*</sup>	30 min <sup>*</sup>
0.2 M Na <sub>2</sub> S <sub>2</sub> O <sub>3</sub>	77.9 ± 7.8	76.9 ± 12.1	62.7 ± 4.1	66.9 ± 10.4	69.2 ± 15.2	86.2 ± 14.7
0.00025% DS	94.7 ± 5.0	90.9 ± 1.0	84.1 ± 3.2	82.8 ± 4.0	81.5 ± 2.3	100.1 ± 14.4
0.0025% DS	80.1 ± 4.4	70.0 ± 0.3	58.1 ± 7.5	53.7 ± 9.7	54.1 ± 12.0	74.7 ± 13.7
0.025% DS	4.1 ± 2.3	3.2 ± 0.8	3.7 ± 2.7	5.2 ± 4.2	4.8 ± 2.3	3.2 ± 0.5
0.125% DS	1.4 ± 0.3	1.5 ± 0.5	2.0 ± 0.9	1.6 ± 0.8	2.6 ± 1.1	2 ± 0.4
0.25% DS	1.5 ± 0.3	1.7 ± 0.5	2.1 ± 1.2	1.5 ± 0.5	3.2 ± 2.0	1.8 ± 0.3
0.5% DS	1.7 ± 0.2	1.6 ± 0.1	2.3 ± 1.3	1.2 ± 0.4	2.9 ± 1.6	1.9 ± 0.3

For DS values reflect the percent cellular viability relative to macrophage viability in 0.2 M Na<sub>2</sub>S<sub>2</sub>O<sub>3</sub>.

\* For Na<sub>2</sub>S<sub>2</sub>O<sub>3</sub> values reflect the percent cellular viability relative to macrophage viability in PBS; mean ± standard deviation.



**Fig. 1 – DS reduces macrophage phagocytosis. (A) *S. aureus*. (B) *P. aeruginosa*. (C) *A. flavus*. Bars represent the mean  $\pm$  standard deviation of three independent experiments. \* $P < 0.05$ .**

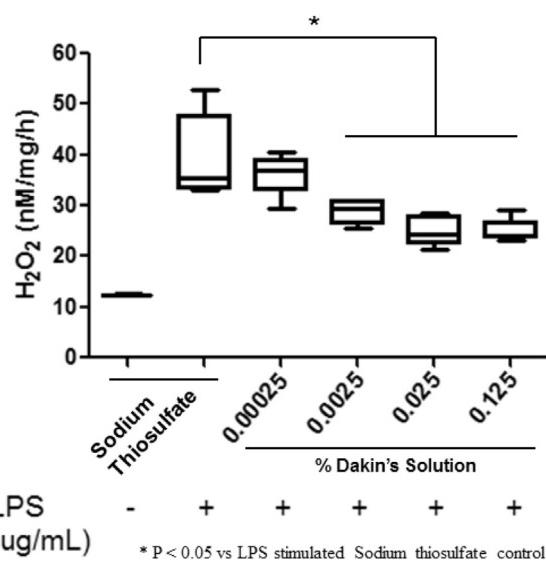
quantitative real time polymerase chain reaction for markers of M1 macrophage activation, including (IL 1 $\beta$ ), inducible nitric oxide synthase 2, and interferon  $\gamma$ , demonstrated an increase in gene expression, with 0.00025% DS having the most pronounced effect. In contrast, genes associated with M2 macrophage activation, including arginase (Arg1), (IL 10), and (IL 4), were not observed to increase to levels similar of the genes associated with classical macrophage activation after treatment with DS.

#### 4. Discussion

We have demonstrated that DS at clinically used concentrations (0.025%–0.25%) was detrimental to macrophage survival and function in vitro. Additionally, treatment with DS resulted in classical macrophage activation. Understanding the impact of DS on macrophages is important as they are key mediators in pathogen defense and are central players in the wound healing cascade [25–28].

It follows that depletion of macrophages in the wound microenvironment by DS may result in impaired pathogen clearance and delayed healing. In addition, we were also able to demonstrate that the remaining macrophages did not have adequate functional activity. *In vivo*, mice depleted of macrophages before injury typically show a defect in epithelial growth, granulation tissue formation, angiogenesis, wound cytokine production, and myofibroblast associated wound contraction [25]. Macrophage depletion during the post inflammatory phase of sterile wounds has also been found to delay wound healing and support hemorrhage because of persistent apoptosis of endothelial cells and detachment of the neuroepithelium [34].

Our study is consistent with other in vitro studies in the literature as DS concentrations  $\geq 0.0025\%$  cause significant cytotoxicity to many cell lines [12–20]. In addition, we were able to demonstrate similar decrements in macrophage function with DS exposure, as have been demonstrated in neutrophils [16–20]. However, it appears that at least in in vitro studies, leukocytes are more sensitive functionally to DS as neutrophil chemotaxis was inhibited with DS concentrations



**Fig. 2 – Treatment of LPS-stimulated macrophages with DS impairs ROS ( $H_2O_2$ ) production. Box plots represent the mean of three independent experiments. Error bars represent the standard deviation.**

**Table 2 – Macrophage adherence following treatment with sodium thiosulfate ( $\text{Na}_2\text{S}_2\text{O}_3$ ) control or DS.**

Concentration	15 min	30 min
0.2 M $\text{Na}_2\text{S}_2\text{O}_3$	18.9 ± 6.8	40.9 ± 8.5
0.00025% DS	12.5 ± 13.9	51.7 ± 14.1
0.0025% DS	5.3 ± 3.6	62.0 ± 9.6
0.025% DS	44.4 ± 13.0	33.1 ± 9.9
0.125% DS	66.7 ± 18.8	54.3 ± 15.7

Values reflect the mean AI; mean ± standard error of the mean.

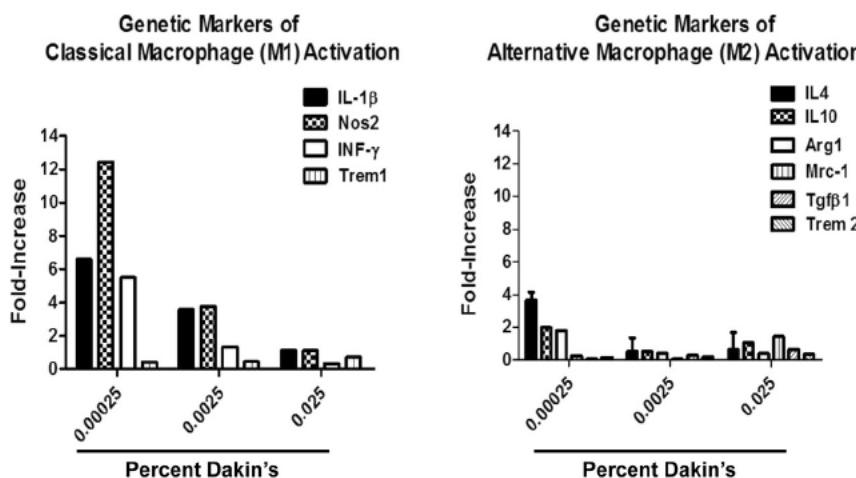
\* $P < 0.05$  compared with sodium thiosulfate control.

>0.000025% [16]. Our results in macrophages were similar as significant dilution was required (0.00025% of DS) to preserve functional capacity. There are only two other studies evaluating the impact of DS on macrophages, and these were designed to evaluate higher concentrations of DS (5.25%) used as a dental irrigant [22,23]. These studies did not address viability, phagocytosis, and ROS production, but macrophage adhesion was noted to be impaired [22,23]. In contrast, we demonstrated that macrophages treated with the higher concentrations of DS (>0.0025%) had significantly increased adherence, whereas lower concentrations ( $\leq 0.0025\%$ ) resulted in slightly reduced adherence. These differences may be related to methodology and/or the different concentrations used. Alternatively, the increased adherence observed in surviving cells in this study with DS concentrations >0.0025% is likely related to macrophage activation secondary to the toxic effects of DS. In contrast, to nonactivated macrophages, activated macrophages undergo significant changes in morphology and the gene expression, enabling them to better adhere to surfaces.

Interestingly, we were able to demonstrate that exposure of macrophages to DS  $\leq 0.0025\%$  resulted in induction of the proinflammatory and M1 macrophage phenotype. This may be an important immune modulating effect to favor pathogen clearance and counteract certain methods of immune evasion. Inducing the M1 phenotype may have particular

value in burn patients. The leukocyte zone of burn wounds have been shown to form 1–2 wk after burn, which is much delayed compared with typical wounds and is in accordance with the clinical observation of increased infection risk within the first or the second week after burn in major burns [35]. Burn subeschar fluid also has the capacity *in vitro* to inhibit cell mediated immune function and polymorphonuclear leukocyte apoptosis [35]. In addition, the majority of thermally injured hosts are shown to be carriers of alternatively activated, M2 macrophages [36]. Thus, it may be possible to use DS in a nontoxic concentration, not only as an antiseptic, but to remove immunosuppressive wound exudate, and induce the M1 phenotype, resulting in a more rapid return of local innate immunity.

Finally, it is worth noting that there are discrepancies in the degree of toxicity of DS when comparing the many *in vitro* studies with the few *in vivo* study results. In *in vitro* studies, DS is not significantly cytotoxic at concentrations ranging from 0.005%–0.025% [12–20]. In contrast, in the *in vivo* studies DS does not appear to alter basal cell viability, tensile wound strength, and reepithelialization at concentrations up to 0.25% [13,14,37]. Interestingly, in one study wounds treated with DS also showed significantly increased neodermal thickness when compared with the controls, and greater numbers of proliferating fibroblasts [38]. Recently, in a small case series of military trauma patients, 0.025% DS was delivered with negative pressure wound therapy as an adjunct to aggressive debridement, and antifungals with clinical success [39]. The discrepancy between *in vitro* and *in vivo* studies is likely related to inactivation of sodium hypochlorite by the plasma components of the wound exudate, necessitating higher concentrations for *in vivo* efficacy, which was known by Carrel and Fleming [10,11]. Carrel hypothesized that because DS did not penetrate deep into tissues, leukocyte function could continue where DS could not penetrate [10]. A potential concern with the use of more dilute concentrations of DS may be loss of a degree of bactericidal activity. For example, it has been demonstrated that dilution of DS  $\leq 0.0125\%$  was effective



**Fig. 3 – Exposure of macrophages with DS results in classical macrophage activation. Gene expression of classical macrophage activation (IL-1 $\beta$ , Nos2, and interferon- $\gamma$ ) and alternative macrophage activation (Arg-1, IL-10, and IL-4) were evaluated by quantitative real-time polymerase chain reaction. Data are presented as fold increase relative to control. Bars represent the mean ± standard deviation of three independent experiments.**

against gram positive organisms, but may allow survival of gram negative organisms (13). However, molds appear to be susceptible to concentrations as low as 0.00025% (21). Thus, the optimal concentration, exposure time, and duration of therapy for clinical use needs to be determined via detailed *in vivo* experiments. Such studies should compare irrigation, soaked gauze application, the Carrel–Dakin method, and negative pressure wound therapy with DS instillation. These studies should also involve close histopathologic evaluation, and extraction of cell lines from the wound bed (fibroblasts, neutrophils, macrophages, and so forth) to evaluate for cyto toxic and functional impacts of therapy. Another approach to explore would be use of higher concentrations of DS initially when wounds have a high amount of purulence and bacterial burden, followed by downward titration of the DS concentration as the wound becomes more sterile to limit potential tissue toxicity [17].

## 5. Conclusions

In conclusion, we have demonstrated that DS at clinically used concentrations (0.025%–0.25%) was detrimental to macrophage survival and function *in vitro*. Additionally, treatment with DS resulted in classical macrophage activation. Understanding the impact of DS on macrophages is important as they are key mediators in pathogen defense and are central players in the wound healing cascade, and depletion by DS may result in impaired pathogen clearance and delayed healing. However, the true impact of DS on macrophage function and clinical wound outcomes will need to be determined in future *in vivo* and clinical studies.

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## Disclosure

The authors report no proprietary or commercial interest in any product mentioned or concept discussed in this article.

## Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.jss.2014.07.019>.

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